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METAL TOLERANCE OF *RHIZOBIUM MELILOTI* ISOLATED FROM HEAVY-METAL CONTAMINATED SOILS

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Summary—Soil populations of rhizobia have been reported to resond to the presence of high concentrations of heavy metals by the acquisition of tolerance to specific metals. To examine this possibility, alfalfa plants (*Medicago sativa*) were collected from soils containing low to very high concentrations of the metals, Cd and Zn. The source of the metals was a Zn smelter in operation for nearly 100 yr. Fifty isolates of *Rhizobium meliloti* were collected and purified from each soil. All isolates, regardless of their origin, were capable of growing on media containing very high concentrations of the heavy metals, Zn, Cu, Ni and Cd. For example, *R. meliloti* isolated from soil with extractable [10 mM $Ca(NO_3)_2$] Zn and Cd concentrations of 0.025 and 0.003 $\mu g\ g^{-1}$ were tolerant of medium concentrations of 162 μg Zn g^{-1} and 22 μg Cd g^{-1}, respectively. There was no correlation between extractable soil metal concentrations and the ability of the isolates to tolerate metal salts in their growth medium. In fact, the greatest number of metal-tolerant rhizobia were isolated from soil containing the lowest metal concentration. These results indicate that the intrinsic level of metal tolerance of *R. meliloti* is much higher than metal activities in soil, even highly contaminated soils. This intrinsic level of metal tolerance probably explains the lack of metal response by rhizobia collected from these soils.

INTRODUCTION

Inputs of heavy metals into soils have been reported to affect microbial populations in soil as well as the processes mediated by these organisms. Population changes in soil, water and sediment contaminated with heavy metals have been observed.

McGrath *et al.* (1988) reported that white clover (*Trifolium repens* L.) grown on metal-contaminated, sludge-amended soil, exhibited reduced yields and N content. Nodule isolates of rhizobia from the plants were subsequently demonstrated to be completely ineffective in plant infection tests (Giller *et al.*, 1989). Similar results have been reported by Rother *et al.* (1983), who showed that N_2 fixation by white clover grown on metal-contaminated soils was reduced. Adverse metal effects on nodulation and plant growth have also been reported for red clover (*Trifolium pratense* L.) by McIlveen and Cole (1974).

Conversely, Kinkle *et al.* (1987) failed to detect significant changes in populations of *Bradyrhizobium japonicum* in sludge-amended soil. No changes in serogroup distribution or in the metal-tolerance of the rhizobial isolates were noted. Field studies by Heckman *et al.* (1987) failed to detect adverse changes in either plant growth of N_2 fixation in the same sludge-amended soils. Borges and Wolluma (1981) added Cd salts to soil and reported that N_2 fixation was not affected.

As the result of the discrepancy in reported results, the current study was initiated. Soils were collected from a metal contaminated region where the source of the metals was a nearby Zn smelter (Beyer, 1988). Soil metal concentrations varied with distance from the smelter. Because metals have been emitted from the smelter for nearly 100 yr, metals in the soil are generally in equilibrium with the soil solution and provided an opportunity to examine extremely contaminated soils in which the metal activities were stable. The use of these soils avoided solubility and activity considerations related to soils amended with recent additions of metal salts or sewage sludge. When metal salts are added to soil, the metal ions are often not in equilibrium with the soil solution, thus resulting in an overestimation of metal effects. Further, additions of metal salts to soil can acidify soil, thus enhancing metal activity. If sludge-borne salts are the source of the metal, problems and questions related to binding and chelation of the added metal may make the results difficult to interpret.

To investigate the relationship between heavy metals in soil and metal effects on soil microorganisms, the following study was initiated. The objective of the study was to determine the maximum metal concentrations that *R. meliloti* can tolerate in artificial media and to compare media concentrations to concentrations found in polluted and non-polluted soil.

MATERIALS AND METHODS

Four soils (A, C, D, F) were collected from the rooting zone (0–15 cm) of alfalfa in an area in close proximity (within 1–2 km) to a Zn smelter located at Palmerton, Pennsylvania. An additional sample (M) was collected from an alfalfa field located in Clarksville, Maryland. This soil had received no exogenous input of metals and served as a control. Soil pH and cation exchange capacity (CEC) were

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Table 1. Total (Aqua Regia) and extractable concentrations of metals in soils from which *Rhizobium meliloti* was isolated

		Zn			Ni			Cu			Cd		
Soil	pH	AR*	CN†	H_2O‡	AR	CN	H_2O	Ar	CN	H_2O	AR	CN	H_2O
							($\mu g\ g^{-1}$ dry soil)						
A	6.15	1540	0.75	0.4	25.4	ND§	ND	31.8	ND	ND	32.4	0.033	0.012
C	6.34	650	0.22	0.14	14.7	ND	ND	22.2	ND	ND	8.63	0.019	0.004
D	6.38	728	0.09	0.08	24.4	ND	ND	18.8	ND	ND	11.6	0.027	0.004
F	6.38	492	0.06	0.07	19.0	ND	ND	40.3	ND	ND	6.57	0.025	<0.001
M	6.44	129	0.025	0.024	12.5	ND	ND	13.4	ND	ND	0.49	0.003	<0.001

*AR = Aqua Regia.
†CN = 10 mM $Ca(NO_3)_2$.
‡H_2O = distilled H_2O.
§ND = not determined.

extremely close for all soils. All soils were maintained at their original moisture content at room temperature. Total metals were extracted with Aqua Regia (McGrath *et al.*, 1988). Five grams of field moist soil was refluxed with concentrated HNO_3 and Aqua Regia, and the extract redissoled in 1.0 M HNO_3. Biologically-relevant extractable metals were measured by shaking 10 g (equivalent dry wt) field moist soil for 2 h with 20 ml deionized water or 10 mM $Ca(NO_3)_2$, filtering, and acidifying the extract with conc. HNO_3 before analysis. All extracts were analyzed by atomic absorption spectrometry, with deuterium background correction for Cd and Zn. Results were corrected for metals in the extracting solutions. Soil pH was measured after 1 h in a 1:1 soil–water suspension.

Alfalfa plants were collected from each site to obtain >50 root nodules. Plants were immediately returned to the laboratory, where *R. meliloti* was isolated from the nodules. Nodules were surface sterilzed in ethanol (95%) and sodium hypochlorite (5.25%), squashed in a saline buffer, and streaked onto yeast extract–mannitol medium (YEM) (Vincent, 1970). Isolates were restreaked onto three additional plates of YEM containing Congo red to ensure purity. All isolates were maintained on YEM slants at 4°C.

Metal tolerance of all isolates was determined on the HEPES (*N'*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–MES [2-(*N*-morpholino) ethanesulfonic acid] (HM) minimal medium of Cole and Elkan (1973). The medium was solidified with 1.5% agar, and the pH was adjusted to 6.6 with NaOH. Filter-sterilized L-arabinose was added after autoclaving to a final concentration of 0.1%. Metals (as $\mu g\ g^{-1}$ ionic metal) were added as $CdCl_2\ 2H_2O$, $CuCl_2\ 2H_2O$, $NiCl_2\ 6H_2O$ and $ZnCl_2$. Metal stock solutions were autoclaved separately and added with arabinose to molten HM medium. Individual isolates were streaked onto each of the metal-amended plates and kept for 5 days at 28°C. The highest concentration of each metal that supported the growth of rhizobia was defined as the maximum resistance level (MRL).

Each isolate was also tested for its ability to nodulate alfalfa and for its influence on plant growth and N accumulation. Ten alfalfa seeds were surface sterilized (5 min in 5.25% sodium hypochlorite), sown into sterilized perlite contained in 500 ml sterile pots and 1 ml pot^{-1} of a log-phase culture of each isolate was inoculated. Pots were placed in the greenhouse and supplemented with artificial light to achieve 12 h light day^{-1}. Plants were watered with an N-free nutrient solution. After 6 weeks of growth, the plants were harvested, and the dry weight of 10 plants determined. Plants were then ground in a Wiley mill and the shoot N-content determined using a C-N-H analyzer (Leco Corp., 1985). Presence or absence of root nodules was noted.

RESULTS

Total metal concentrations in soil varied with the origin of the soil (Table 1). The greatest differences in concentration were observed for Cd and Zn, as these were the primary metals discharged from the smelter. Concentrations of total Zn and Cd in the most contaminated soil (A) were 12- and 66-fold higher than in the control soil (M).

Water and $Ca(NO_3)_2$ extractable metals were also examined in each soil. In all cases, the extractable metal concentrations were only a small fraction of the total metal concentration, demonstrating that these metals were in equilibrium with the soil solution. The great majority of metals were chelated, complexed or precipitated (in or on the solid phase), resulting in very low extractable concentrations, even though total concentrations were often very high. Higher

Table 2. Mean ($\bar{X}$) maximum metal resistance level, MLR range (R), and coefficient of variation (CV) of *Rhizobium meliloti* (50 single isolates per soil) from metal-contaminated soils

	Maximum resistance level											
	Cu			Zn			Ni			Cd		
Soil	$\bar{X}$ ($\mu g\ g^{-1}$)	R	CV (%)	$\bar{X}$ ($\mu g\ g^{-1}$)	R	CV (%)	$\bar{X}$ ($\mu g\ g^{-1}$)	R	CV (%)	$\bar{X}$ ($\mu g\ g^{-1}$)	R	CV (%)
F	3.52	2–4	17	10.4	10–20	19	11.9	7–13	18	15.4	10–25	19
A	2.24	1–4	45	16.2	10–30	44	9.00	6–16	27	15.4	8–20	15
C	3.82	1–5	33	25.4	10–40	43	11.4	7–13	22	20.3	9–25	26
D	3.72	2–6	28	35.4	20–40	18	12.1	8–13	15	21.8	14–25	22
M	9.30*	1–18	75	162*	10–175	45	13.4	3–25	31	21.9	11–35	36

*Significantly different from other soils at the 5% level of probability.

concentrations were found with 10 mM $Ca(NO_3)_2$ extracts than with water extracts, probably as a result of competition for metal binding sites on the soil.

The metal tolerance of isolates from the five soils is presented in Table 2. In general, Cu was the most toxic, while Zn was the least toxic. The only exception to the pattern occurred with soil F, where Cd was the least toxic of the metals. Specific metal toxicities were similar to previous studies with *B. japonicum* (Kinkle *et al.*, 1987).

For all metals, except Zn, MRL averages were quite similar for all isolates from Palmerton. For Zn, while differences in the MRL of isolates from Palmerton of over 3-fold were observed, the high degree of variability prevented the detection of significant differences. A significant difference between isolates from soil M (Clarksville, Maryland) and isolates from the contaminated soils of Palmerton was noted for Cu and Zn, where the average difference between Palmerton and Clarksville isolates was three and seven times, respectively. No significant differences were observed for Ni and Cd.

These results demonstrate that there was no correlation between the concentration of extractable metals in the soil and the MLR of the isolates. Even when large differences in extractable metal concentrations were present, MRLs did not vary with soil metal concentrations. In fact, the highest MRLs were found in soil M from Clarksville. This soil had much lower concentrations of metals than soils from Palmerston.

The MRL values were most variable in soil M which had the lowest metal concentrations. Relative low levels of variability in the contaminated soils and the high variability in the noncontaminated soil is reflected by the coefficient of variation (CV) and the range of MRL values. This is most obvious in soil M where the CV was over two times that of the average of the other soils. Generally, most values were closely grouped around the mean, except for a few low values. For soil M, the maximum MRL observed for Zn was 175 $\mu g\ g^{-1}$ and the average MRL was 162 $\mu g\ g^{-1}$. However, approx. 10% of the total number of isolates in this soil exhibited MRLs of 10–15 $\mu g\ g^{-1}$.

A comparison of the MRLs in Table 2 with metal concentrations in Table 1 demonstrates that there was little correlation between these two variables. However, it should be noted that total metal concentrations have little relation to metal effects on microbes, as numerous factors affect solubility and, thus, activity of the metal. Haq *et al.* (1980) studied the use of various extractants and compared these to actual metal uptake by plants. Although there was a significant degree of variation between the various extractants, the two extractants used by us gave a reasonable estimate of metal activity. If extracted concentrations are assumed to be representative of actual metal activities, it is apparent that the differences between concentrations of metals in the HM medium and soil metal activities are orders-of-magnitude different from one another. For example, the average Cd MRL was 22.4 $\mu g\ g^{-1}$, while the average extractable soil concentration [$Ca(NO_3)_2$] was 0.021 $\mu g\ g^{-1}$. This represents greater than a 1000-fold difference between "available" metal concentrations and the ability of the isolates to tolerate Cd. Similar differences between metal activites in soil and the intrinsic ability of all isolates to grow in the presence of the metals were noted for all metals studied.

Not all of metals in the HM medium are available to affect isolates growing on the surface. Angle and Chaney (1989) have demonstrated that more than 50% of the Cd added as a salt to HM medium was removed from solution. The MRL, as determined on HM medium, would thus be somewhat higher when compared to activities estimated in soil by the two extractants. This difference, however, is relatively small in comparison to the orders-of-magnitude difference noted between the MRLs and extracted metal concentrations.

The MRL data described above indicate that there was no correlation between soil metal concentrations and the ability of the isolates to tolerate the presence of metals in their environment. This was further confirmed by data on plant and N accumulation. There were no significant differences in the dry weight or N content of any plants inoculated with isolates from each of the soils. The average N content for all plants across all soils was 3.6%. Total shoot N (percent N % plant dry weight) was also not significantly different for any plants inoculated with isolates from each of the soils. Coefficients of variation within each soil were low and averaged 16, 5 and 15% for shoot weight, shoot percent N and total shoot N, respectively.

DISCUSSION

Metal deposition into soil over long periods results in high total metal concentrations, but low concentrations of available metals. Metals are removed from solution by ion pairing, precipitation, and sorption onto colloid surfaces. Metals that have been precipitated or chelated (DeKock and Mitchell, 1957; Halvorson and Lindsay, 1977) are not active in the soil solution and thus have no effect on plants or microbes. For most metals, only a very small fraction of the metal in soil is soluble and, thus, affect the soil population of *R. meliloti*. Therefore, although the soils in our study were considered to be highly contaminated, anticipated effects on the soil microbial population did not develop.

Rhizobium meliloti grow in the presence of concentrations of metals that were much higher than metal activities in soil. Although a portion of the metals in the HM medium was removed from solution by a number of chemical processes, the difference between extractable metal concentrations and the MRL was so great that there was little doubt that metals in the soil were not exerting an antagonistic effect on *R. meliloti*. For example, the MRL values for Cd and Zn were approx. 1000- and 200-fold greater, respectively, than extractable [$Ca(NO_3)_2$] concentrations of these metals in soil. These "orders-of-magnitude" differences between soil metal activity and MRLs essentially eliminate any potential effect on the soil population of *R. meliloti*.

Microorganisms tolerant of metal contaminants are known to have two mechanisms by which they protect themselves from the toxic effects of heavy metals. Exclusion, as mediated by the extrapolysaccharide capsule around the cell, has been shown to

prevent cellular uptake of metals (Bitton and Freihofer, 1978; Mitra *et al.*, 1975). The polysaccharide capsule around most species of rhizobia is quite thick and therefore should provide adequate protection against most metals. Secondly, several genera of soil microbes are known to produce phytochelatins, which may internally bind metals and thus effectively lower activity within the cell. Phytochelatins are low molecular-weight, cysteine-rich peptides that bind specific metals (Grill *et al.*, 1986; Higham *et al.*, 1984).

Our findings are in contrast to the results of several other studies, including those of McGrath *et al.* (1988) and Giller *et al.* (1989). These authors reported that N_2 fixation and growth of white clover in a sludge-amended soil were reduced, most probably by the sludge-borne metals. We detected no such inhibition, and several previous papers have shown an increase in rhizobial numbers, nodulation and nitrogen fixation (Heckman *et al.*, 1987; Kinkle *et al.*, 1987) when legumes were grown in sludge-amended soil. Several possible explanations are suggested for the discrepancy between studies. First, McGrath, Giller and coworkers have primarily studied white clover, whereas our studies have examined alfalfa and soybeans. It is possible that the macrosymbiont hosts differ in their symbiotic capacities when grown under stressed conditions. White clover may be more sensitive to the toxic effects of heavy metals, thereby failing to achieve an effective symbiotic relationship with *R. leguminosarum* bio. *trifolii*. Secondly, the microsymbionts may differ in their sensitivity to the toxic effects of heavy metals. This could be determined by comparing the MRI of diverse isolates of *R. leguminosarum* bio. *trifolii* and *R. meliloti*. It is also possible that *R. leguminosarum* bio. *trifolii* in the U.K., which were the subject of the work by McGrath, Giller and coworkers, are local isolates that were less tolerant of high heavy metal concentrations when compared to the same biovar from other areas of the world. Adaptation of isolates to local conditions is possible and could result in divergent lines of the same species. This possibility is supported by the observation that the MRLs between the isolates from the Palmerton and Clarksville locations were significantly different from one another, although the difference was not related to metal pressure.

We have found that for alfalfa, there is little relationship between heavy metal concentrations in soil and the growth and survival of *R. meliloti*. No correlation between MRLs and soil metal activity was observed, despite the fact that soil metal concentrations ranging from low to very high were examined. The lack of a significant relation occurred because all isolates of *R. meliloti* examined were intrinsically tolerant to very high concentrations of metals.

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